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#16
6-28-95
C. Brown

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:	John B. Fenn, et al
Serial No.:	07/911,405
Art Unit:	2506
Examiner:	Kiet T. Nguyen
Filing Date:	7/10/92
For:	Multiply Charged Ions
Docket # Prev. Atty.	840.004 DIV

Hon. Commissioner of Patents & Trademarks
Washington, DC 20231

Response to Office Action of October 12, 1994

Receipt is acknowledged of the communication from the Examiner dated October 12, 1994. The Applicants hereby request a two month extension of the time for response to that action and enclose a check for the fee of $185. Also acknowledged is the interview on 2/3/95 kindly granted Applicant John B. Fenn by Examiners Kiet Nguyen and Jack Berman. Discussion at the interview was limited to the question raised by the Examiners on utility of the claimed compositions of matter. That discussion was most helpful in clarifying for the applicant the reasons for that question. As a result he hopes he has been better able to address that question in this response. As promised in the interview this response provides

I hereby certify that this response is being deposited with the U.S. Postal Service as First Class Mail, postage prepaid, in an envelope addressed to Commisioner of Patents and Trademarks, Washington DC 20231 on 6 March 1995.

John B. Fenn 3/6/95

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evidence and arguments in support of the Applicants belief that the multiply charged ions that are claimed as a novel composition of matter have a meaningful existence and utility in and of themselves that are above and beyond a transient role as intermediates in an analytical method.

The Examiner seems to believe that the claimed multiply charged ions are unpatentable as compositions of matter because they lack utility and have only an incidental existence in the course of a procedure for determining molecular weight by mass spectrometry The Applicants respectfully disagree for the reasons that follow.

The Legal Standard for Utility Requires a Very Limited Showing That the Invention Possess Any Form of Usefulness

When an invention meets at least one stated objective, utility under Par. 101 is clearly shown. See, Raytheon Co. v. Roper Corp., 724 F.2d F.2d 951, 220 USPQ 592,598 (Fed. Cir. 1983); Standard Oil Co. (Indiana) v. Montedison, SpA, 664 F.2d 356,375, 212 USPQ 327, 344 (3rd Cir. 1981), cert. denied, 456 U.S. 915 (1982) ("It is recognized that the required utility disclosure can be met if the disclosed properties of the invention indicate the material is useful for a specific purpose.")

For an invention to be non-patentable on grounds of non-utility, it must have simply no usefulness, serving no known or realistic purpose. To be patentable, "a claimed invention must only be capable of performing some beneficial function." E.I. du Pont de Nemours & Co. v. Berkley & Co., 620 F.2d 1247,205 USPQ 1, 8 n.10, 10 n. 17 (8th Cir. 1980)(underline emphasis added; bold emphasis

in the original) See also, Envirotech Crp. v Al George, Inc., 703 F.2d 753,221 USPQ 473,480 (Fed. Cir. 1984) "Lack of utility means the invention is incapable of achieving any of the aims of the patent under any conditions." E.I. du Pont de Nemours & Co. v Berkley & Co., 620 F.2d 1247, 205 USPQ 1, 8 n.10, 10 n. 17 (8th Cir. 1980) (internal quotation marks omitted, underline added). Moreover, "the defense of non-utility cannot be sustained without proof of total incapacity". E.I. du Pont de Nemours & Co. v. Berkley & Co., 620 F.2d 1247, 205 USPQ 1, 8 n.10, 10 n.17,(8th Cir. 1980) "Proof of non-utility or non-operativeness must be strong...every reasonable doubt being resolved in favor of the patentee" Id. (emphasis added.) See also, Envirotech 221 USPQ at 480.

The composition of matter claimed in the present invention clearly has utility. As explained at great length in the original specification the high degree of charge multiplicity on the ions of large molecules in the claimed composition of matter makes possible the accurate determination of the masses of those ions and, therefore, the molecular weights of the large molecules from which they are formed. Not only does this multiple charging make molecular-weight determination possible for molecules much larger than could previously have been "weighed" by any available mass analyzer, it also allows investigators to perform this "weighing" with analyzers whose nominal maximum mass capacity is much below the actual mass of the ions being weighed. (This "nominal" maximum mass capacity is traditionally expressed in terms of the mass of singly charged ionic species because before the invention practically all mass spectrometry was carried out with singly charged ions.)

Thus, for example, as a result of the invention a small relatively inexpensive quadrupole mass analyzer in the inventors' laboratory with a nominal upper limit for mass of 1500 daltons, could weigh protein molecules with masses up to almost 40,000 daltons, heavier than the largest and most expensive magnetic sector instruments could weigh with comparable accuracy before the invention was made.

An additional benefit of the invention is the multiplicity of charge states in the claimed composition of matter, i.e. the wide variation in the number of charges per ion in the ions of a particular parent molecular species. A consequence of this charge state multiplicity is that the mass spectra for the claimed compositions of matter contain a plurality of peaks for each particular parent molecular species, each peak occurring at a different value on the m/z scale of the spectrum. The initial reaction of almost all mass spectrometrists to this peak multiplicity was one of horror because, as many said, "a spectrum with several peaks for each species will be much too complicated for meaningful interpretation. Moreover, dividing the available charge among so many peaks will greatly decrease analytical sensitivity." In fact, because these peaks are coherent, the ions of each peak differing only by one charge from those of immediately adjacent peaks, it becomes easy for a properly programmed computer to identify each one of the multiplicity of peaks that relate to a particular parent molecular species, even though they may be interspersed with peaks due to species other than the parent species of interest. Because each of the several peaks stemming from that parent species constitutes an independent measurement of the parent species mass,

an investigator can average the results of those independent measurements thereby greatly increasing the accuracy of, and confidence in, the resulting value of molecular weight for the parent species. As for the feared dilution of available charge, the inventors showed that appropriate deconvolution algorithms could in effect combine the signals from each of the several peaks for each species, thereby substantially increasing the signal/noise ratio for the single peak in the deconvoluted spectrum that corresponded to a particular parent molecular species.

At the time that the inventors made their discovery nobody had even dreamed that large and fragile molecules like proteins could be transformed into ions having such extensive multiple charging that a single large molecule could become an ion carrying many tens of elementary charges. Indeed, it is doubtful that anybody believed that such ions could even exist, let alone be readily produced. For whatever reason it apparently never occurred to any previous investigators that it might be possible to put large numbers of charges on a single molecular ion, thus reducing its m/z value to the point where simple, relatively inexpensive analyzers could provide accurate molecular weight values for very large molecules. By the same token, they apparently never contemplated the possibility that such extensive multiple charging would also enable large expensive analyzers to provide extremely precise mass measurements for very large molecules. Both of those valuable features have been made possible by the invention.

Accurate molecular weight information is always useful, often necessary, and sometimes sufficient, in determining the identity

of a molecular species. Positive identification of such species plays a vital role in elucidating the chemical processes that govern living systems. It is often a sine qua non in the development of drugs and other therapeutic agents. In biotechnology, for example, investigators often try to synthesize artificial proteins and peptides that may differ from their natural counterparts only by the substitution of one amino acid for another in the sequence of amino acids found in the natural compound. Such syntheses often involve a long and costly stepwise procedure by which amino acids are added one at a time to a growing chain in a selected order. The chance for error increases substantially as the number of steps increases. Thus, when a long sequence has been prepared the investigator wants to know whether the result is what he or she had planned. Because the amount of material produced is very small it is important that the amount of sample consumed in any verification procedure be very small. An accurate determination of molecular weight is a very valuable piece of evidence in any verification procedure. By transforming some of the synthesized material into the claimed composition of matter the investigator can then use mass analysis to obtain a very precise value of molecular weight from a very small quantity of sample and thus learn whether the product is actually what was sought.

In another variation on this theme researchers sometimes spend a great deal of effort in extracting and isolating active species in natural materials of animal or vegatable origin. These active species may be large biomolecules such as proteins, peptides glycoproteins, carbohydrates, or nucleic acids. Moreover, they

may be present only at trace levels in the source material. Consequently, investigators may have to process many pounds of source material by lengthy and complicated extractive procedures to produce a few milligrams of product. It then becomes essential to identify the components of this product while consuming only a very small amount of it. Here again, transformation of few micrograms, or even nanograms, of the product into the claimed composition of matter makes it possible for mass analysis able to provide invaluable information on the product's composition and identity.

These examples are only two of many applications in which the ability of the claimed composition of matter to provide accurate values of molecular weight for large and complex molecules plays an important and valuable role. Thus it should not be surprising that the invention is in daily use by many investigators in many parts of the world. Most of the major mass spectometer manufacturers now offer electrospray sources for their instruments, often because those sources can produce the novel ion populations that are claimed in the subject invention. Last June in Chicago at the annual **Conference on Mass Spectrometry and Allied Topics**, there were over 300 papers involving electrospray ionization. A large fraction of those papers related to the production and mass analysis of large multiply charged ions to determine the molecular weight of their parent species. Clearly, the market place has endorsed the utility of the claimed ion populations in the determination of molecular weight.

The Examiner has posed the question of whether the claimed compositions of matter have a meaningful existence per se or

whether they are simply intermediates having a transient existence during determination of molecular weight by the methods set forth and claimed in the already issued US patent 5,130,538. The method claims in that patent are based on the same specification in the original application of which the subject application is a continuation. The inventors respectfully suggest that the claimed ions are <u>not</u> simply transient species akin, for example, to free radicals and other active intermediates that play an important role in component steps of many chemical reactions. For example, in the apparently simple overall reaction $H_2 + Br_2 \rightarrow 2\ HBR$, it has been well established that H atoms and Br atoms are the so called chain carriers that keep the reaction going at a rapid rate. Thus, $H + Br_2 \rightarrow HBr + Br$ and $Br + H_2 \rightarrow HBr + H$ are the actual reactions by which the product HBr molecules are formed. In this model for the reaction mechanism each formation of a product HBr molecule is accompanied by the generation of an H atom or a Br atom, each of which can then initiate reaction with another Br_2 or H_2 reactant molecule. Thus, once the reaction gets started it can keep going by continuing to produce the "chain carriers" H and Br that make it go until all the Br_2 and/or H_2 molecules have been consumed. Many if not most so-called bimolecular reactions actually take place by way of intermediates such as the H and Br atoms in the simple example just described. These intermediates are indeed transient in the sense that they are usually so reactive that they will undergo a reaction and lose their identity in any collision with any but the most inert molecular species. All the reactive collisions that do not produce an offspring

intermediate result in "terminating the chain", i.e. net destruction of the carrier that produces reaction. Thus, these reactive intermediates rapidly disappear in the absence of reaction partners that will let them reproduce themselves. Even when their reactions are reproductive, the lifetimes of these reactive intermediates are generally so short that their steady state concentrations in reacting mixtures are often too low to be detected directly. In many cases the only evidence for their existence is inferential in that one cannot explain observable features of the reaction system without invoking their presence. In effect they have no meaningful existence except as part of the reaction process.

The situation is quite different for the claimed multiply charged ions. They are produced during the evaporation of charged droplets in a chamber containing bath gas at atmospheric pressure. They drift through this gas toward the aperture (tube or orifice) leading into the vacuum system. There they become entrained in some of the gas that flows into the aperture to emerge as a supersonic free jet in the first of one or more vacuum chambers in sequence. Finally they enter a mass analyzer-cum-detector combination and are weighed. Most analyzer-detector combinations depend upon the destruction of the ion in order to provide an output signal. In the particular case of analyzers based on "Ion Cyclotron Resonance (ICR), the ions can be "trapped" in an orbit cycle in a magnetic field whose cycling frequency is determined by their mass and the strength of the field. As they orbit they repeatedly pass by an "antenna" coil and induce a detectable AC current in an external circuit whose frequency indicates the ion

mass/charge ratio. In principle, the ions could be recovered unscathed after mass analysis has been completed. In practice, it is not worthwhile to attempt such recovery. Nevertheless, multiply charged ES ions have been stored for hours in such traps. A somewhat similar situation obtains in so-called quadrupole ion traps which can store ions for long periods of time by appropriate combinations of oscillating and static electric fields. However, to complete the weighing process requires removal of the ions from the trap for detection on an external detector that perforce destroys them.

Even with "straight through" mass analyzers that do not involve ion storage, the time from formation of ES ions in the source to their destruction can be tens of milliseconds which relatively speaking is eons in a world where the characteristic time scale for molecular interaction events is measured in micro-, nano-, pico- or femto- seconds. Moreover, the formation of ES ions occurs at a time and place that are both different from when and where they are weighed and detected. They are born in a source at high pressure and die in a detector in high vacuum, having passed through an analyzer where they are weighed. During their lifetimes they undergo many millions of collisions with other molecules without losing their integrity or identity. In contrast, the active intermediates in a chemical reaction are produced, used and consumed at the same place and at the same time that the reaction is occurring. They have no existence except in the reaction scene. The ions constituting the claimed composition of matter are produced at a time and place quite different from the time and place of their use (i.e. in the mass analyzer). Thus, they do exist and

have a meaning apart and away from the scene of their role in determining molecular weight. It is true that on the time scale of human experience, the claimed ions lead a very transient existence, but on the time scale appropriate for molecular events they live to a ripe old age. Indeed, as mentioned above, populations of claimed ions have been maintained or stored in ICR traps and Quadrupole Ion Traps for hours at a time. Clearly, their existence can be real and have actual or potential significance or meaning for time intervals much longer than the times that are required for their mass analysis.

It is revealing to recall how the discovery of the claimed multiply charged ions occurred. The Applicants one day injected a solution of bovine insulin in 50:50 methanol water into their electrospray mass spectrometer. The resulting spectrum showed three distinct large peaks on the m/z scale. They knew the molecular weight of this compound was 5,730 and the nominal upper limit of mass for their analyzer was 1500. At first they suspected fragmentation of the parent molecule but consideration of the m/z values persuaded them that the three peaks were due to ions comprising intact insulin molecules with respectively 4, 5 and 6 protons attached. In the next couple of weeks they tried solutions of several other proteins and peptides having molecular weights up to 39,800 (for alcohol dehydrogenase). The number of peaks attributable to the solute species increased with increasing molecular weight, reaching 15 for the largest. The inventors were so excited at obtaining clean and unique mass spectra for such large molecules that they didn't immediately realize all the significance of their

observations. When initial excitement waned they started trying to interpret the spectra and soon found that each of the peaks could always be attributed to a complex comprising a solute protein or peptide molecule with some number of protons attached. Moreover, they found that the peaks always formed a coherent sequence in which the ions of each peak differed from those of adjacent peaks by a single adduct proton. Next they found that knowing the molecular weight of the parent molecule they could determine the number of charges on the ions of each peak if they assumed the right value for the identity (mass) of each charge. At that point they did not yet recognize that these features of the spectral peaks also meant that molecular weight values could be obtained for an unknown parent species. That possibility, along with the realization that such molecular weight values could be extremely accurate, did not occur to the Applicants for another week or two when they suddenly realized that the m/z value of each peak was in effect an independent measurement of the parent molecular weight. Thus, one could average over as many values as there were peaks to obtain a most probable value of molecular weight with great accuracy and confidence.

The point of this account is that the claimed ion populations were discovered and characterized quite some time before their use in determining molecular weight was realized. Thus the claimed ions had an existence and meaning all apart from their use in molecular weight determination which came after their discovery. The use of mass analysis in their discovery and description does not of itself automatically reveal utility in determining molcular weight.

The applicants recognize that future investigators may find

methods other than electropray ionization to produce the unique ion populations they discovered and are claiming. They believe that the claimed ion populations themselves, all apart from the method used to produce them, are sufficiently unique and useful to be entitled to patent protection. The Applicants further believe that under the applicable legal standard for utility they are eligible for patent protection on these novel ion populations without being required to have shown uses for them other than in the determination of molecular weights, an application that they have fully described in the specification. That application is so important and valuable that the claimed composition of matter is already in daily use by many practitioners in many countries.

The applicants recognized that the multiply charged ions they discovered were indeed new and unexpected. They had every reason to expect that new and unexpected uses would be found for these ions because it has very often been the case that novel compositions of matter with unusual properties have turned out to have many uses other than the one to which they were originally put. It would be counter-productive to the interests of society if inventors were to be denied patent protection on new compositions of matter except for the particular use that they initially find, describe and demonstrate. Such a policy would discourage and delay disclosure of discoveries by inventors until they themselves had explored, demonstrated and evaluated as many other uses as possible for their invention. The resulting delays in disclosure would mean that society would be denied the fruits of an invention for however long it might take the inventors themselves to obtain the time and

and resources required to develop other uses for the invention. Only then could the inventors gain enough protection to make disclosure of their invention worthwhile. After all, the fundamental purpose of patent policy is to encourage the prompt disclosure and dissemination of discoveries and inventions by providing protection and resulting economic benefits to the inventors.

In this perspective it is interesting to reflect on additional uses for the multiply charged ions of the invention that already have been found by other investigators as a result of the applicants' discovery. Some of these other actual and possible uses are set forth briefly in accompanying exhibits A through F which will be summarized briefly in what follows.

EXHIBIT A is a paper in the Journal of the American Chemical Society by Chowdhury, Katta and Chait showing for the first time that the charge state distribution in multiply charged electrospray ions depends upon the conformation of the parent molecules in solution. This discovery introduced the possibility that measuring the charge state distribution of ES ions may provide a new approach to obtaining information on the conformation in solution of proteins, and possibly other kinds of molecules. Access to such information is of great interest and importance because the conformation of species like proteins determines their biological activity. This seminal discovery from the Chait group has stimulated exciting and fruitful studies by many other investigators, one of which is described in Exhibit B. Although mass spectrometry of multiply charged ions is the key investigative tool in these conformation studies, the explicit determination of molecular weight is not one

of the objectives, nor is it always necessary.

EXHIBIT B comprises the abstract and first page of a paper in the Proceedings of the National Academy of Science that resulted directly from the findings of Exhibit A. It shows that the rate and extent of hydrogen-deuterium exchange for electrospray ions in the gas phase seems to depend on the conformation of the parent species in solution. This result provides a perspective on the important problem of protein conformation that supplements the results described in Exhibit A. The very idea that the effects of conformation on the reaction kinetics of proteins could be studied in the gas phase was almost inconceivable before the applicants made their invention. This "impossible dream" became reality as a direct result of the properties of the compositions of matter that they disclosed and claimed in the subject application.

EXHIBITS C,D and E show abstracts of papers or presentations from the Sundqvist group at Uppsala University. They have been using multiply charged electrospray ions as projectiles for bombarding surfaces. The extensive multiple charging of these ions means much higher charge/mass ratios, and therefore translational energies, could be obtained for much more massive particles than had been possible before the inventors made and disclosed the claimed compositions of matter. There is little doubt that some scientifically interesting results will be obtained from these studies of particle surface interactions at very high incident energies. Indeed, some ultimately practical benefits might be achieved, one of which is outlined next.

EXHIBITS F AND G. In trying to achieve thermonuclear fusion

(D + D -> He) by inertial confinement. ultra powerful lasers are used to dump a lot of energy in a very short time uniformly over the surface of small solid pellet of deuterium. The resulting rapid evaporation of material from the surface produces a reactive implosion that results in tremendous compression of the core of the pellet. In this way investigators hope to achieve temperatures and densities in the pellet interior that are high enough to produce thermonuclear fusion. This approach has a severe handicap in that the laser energy is transformed into translational energy of light electrons which must then transfer it to the much heavier nucleons, an inherently inefficient process. Moreover, radiation by the energetic electrons results in substantial energy loss. As indicated in the publications of EXHIBIT F, the Brookhaven Group had the idea of bombarding a solid target with highly accelerated cluster ions comprising a hundred or so D_2O molecules around a D_3O^+ ion. The resulting translational energy of the cluster is mostly in in the nucleons of the D atoms rather than the much lighter electrons. Upon stagnating at the target surface that translational energy would be thermalized to produced high translational temperatures of those nucleons. The stagnation would involve shock waves in the cluster which the investigators hoped would provide peak temperatures at local hot spots that could reach fusion conditions. Because the transfer of energy from heavy deuterons to light electrons is just as inefficient as the transfer from electrons to deuteron nuclei, the electrons would not be rapidly excited so that radiation losses should be smaller. After some preliminary experiments that looked promising the group finally decided that

the yield of true thermonuclear fusion events was much lower than the first results with these heavy water clusters had indicated. The Yale group meanwhile had shown that intact ES ions could be produced from polyethylene glycol oligomers with molecular weights of 5,000,000, a thousand times heavier than the cluster ions used by the Brookhaven Group. Moreover, the charge/mass ratio for the large oligomer ions was two to three times greater than for the cluster ions. These characteristics were very attractive to the Brookhaven group so they fitted an ES source to their accelerator and obtained some preliminary experiments on surface bombardment with large ES ions that are described in EXHIBIT G. Then funding problems shut down the experiments and no more work has been done. However, the underlying logic of this approach seems reasonably sound and the ability of ES ionization to produce very large ions with high charge/mass ratios might make it work. Of course the parent oligomers would have to be deuterated for fusion to be effected but such deuteration is not a serious problem. The experiments of EXHIBITS C,D,E,F and G show uses for the claimed composition of matter that are markedly different from molecular weight determination and could be of much greater economic significance if they led the way to fusion power generation! The Uppsala group is continuing their experiments and may well pursue this fusion possibility further. The Brookhaven group would like to carry out further studies if they can obtain funding.

EXHIBITS H AND I comprise two abstracts, H by Kolli and Ron Orlando on the use of ES ions for sequencing peptides and proteins, and I from the McLafferty group on sequencing oligonucleotides.

Of major importance in bio science and technology is the problem of determining the sequences in which amino acids are linked together in peptides and proteins as well as the order in which nucleotide base pairs are linked in DNA. Most previous work on this problem has been carried out by wet chemical methods that are relatively slow and tedious compared to what might be achieved by tandem mass spectrometry or MS-MS. In this latter technique a first analyzer selects a particular parent ion (e.g. a protein or peptide). The selected ion is then dissociated into fragments by collisions with inert gas molecules such argon. A second mass analyzer determines the masses of the fragments. For example, suppose a small peptide ion is represented by ABC+ where A, B and C are three different amino acid residues. If the fragment ions include A+, B+, C+, AB+ and BC+ but no AC+ then one can be sure that the order of linking in the original peptide was indeed ABC. If there were AC+ ions but no AB+ ions the sequence would have to have been ACB+. The advent of multiply charged ions discoverd and claimed by the applicants provided the exciting possibility of extending this approach to much larger parent molecules. Moreover, the multiple charging means that one parent ion can give rise to several offspring ions. Singly charged parent ions can give rise to only one offspring ion. For large primary ions with extensive multiple charging there arises the problem of determining the charge state of multiply charged offspring ions because there is not the coherent sequence present in fragment ions that is always present in the initial ion populations that are produced by ES. However, as the abstracts show, mass analyzers having sufficiently

high resolution can provide the charge state of any ion species from the spacing of the isotopically resolved peaks. In sum, the multiply charged ions of the claimed composition of matter have given rise to a rapidly expanding activity in mass spectrometric sequencing of biopolymers. Of course the determination of mass is an important component of the method but to achieve the overall objective of sequence determination that the claimed multiply charged ions make possible involves much more than just mass measurement and the primary multiply charged ions play a much more complex and active role than to serve simply as inert subjects for mass analysis.

EXHIBIT J is the abstract and first page of a paper by Covey and Douglas in the Journal of the American Society for Mass Spectrometry. It shows the use of multiply charged ions in measurements of collision cross sections which give information on the effective geometric size of these ions in energy loss collisions. This study is another example of the use of multiply charged ions other than in the determination of their molecular weight.

EXHIBIT K is the abstract and first page of a paper by Wysocki et al in the Journal of the American Society for Mass Spectrometry. It describes results obtained in the dissociation of multiply charged ions by collisions with a surface. Such Surface Induced Dissociation (SID) is likely to play an important role in Tandem Mass Sectrometry or MS-MS as outlined in the above discussion of EXHIBITS H and I. It is one more use of multiply charged ions that transcends the mere determination of molecular weight.

Conclusion. The Applicants believe that the foregoing discussion along with EXHIBITS A through K constitute strong evidence for the utility of the claimed multiply charged ions over and above their use in determining molecular weight. The Examiner has suggested that they are simply transient intermediate species that have only an evanescent existence in the course of such molecular weight determination. He seems to believe that the claimed multiply charged ions are analogous to the reactive intermediates in chemical reactions that often have only a transient existence while the reaction takes place and cannot exist outside of the reaction zone. As pointed out in the above discussion, the ions of the claimed composition of matter are produced well before the mass analysis takes place, in a region, at a time, and under conditions that are entirely different from those where the weighing takes place. Already in existence in the source, they are then transported into the mass analyzer and thence to the detector. In the apparatus described in the application (an electrospray source combined with a simple quadrupole mass analyzer) they have lifetimes and stabilities that are orders of magnitude greater than the intermediates of a chemical reaction. Moreover, as it has subsequently turned out, these multiply charged ions can exist for many hours in traps based on ion cyclotron resonance or quadrupole fields.

With respect to the question of utility, the Applicants believe that the ability of the claimed ion populations to provide molecular weight values for much larger species with much greater accuracy than had been possible before the invention, is in itself sufficient utility under the applicable legal standard to justify allowance

of the claims. To be remembered, as recounted above, is that the multiply charged ion populations that are claimed as new compositions of matter were discovered and characterized before their utility in determining molecular weight was realized and reduced to practice. In that discovery process a mass analyzer was used to detect and reveal some features of these new and unusual ion populations but not to determine the molecular weight of the species from which they were formed in those first experiments. The utility of these claimed populations in determination of molecular weight was discovered later in a separate study. Thus, the claimed ions had a meaningful existence separate and apart from and prior to their use in determining molecular weight. Their existence was not and is not simply an incidental intermediate in the determination of molecular weight.

In addition, the Applicants have shown in the accompanying Appendices that many other uses for the claimed ions have emerged. To be sure, these other uses were not known and set forth in the specification and of course cannot be claimed as such, but some if not most of them constitute, for the claimed ions with multiple charges, uses to which experimentalists have often put the singly charged ions with which they have long been familiar. The Applicants had every reason to believe that the novel multiply charged ions that they discovered and have disclosed in their application, along with a method of producing them, would be used by other experimentalists in the various well known ways that singly charged ions have long been used, once those investigators learned about the invention. Indeed, just that has happened.

In sum, the Applicants believe:

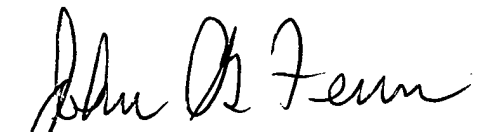
(1) that they have discovered and characterized a new composition of matter comprising populations of multiply charged ions as fully described in the application,

(2) that they have demonstrated the utility of this new composition of matter in achieving the valuable objective of obtaining accurate values of molecular weight with more accuracy and for much larger molecules than was possible prior to their invention,

(3) that the claims as presented define a patentable advance over the prior art of record and

(4) that the application is in condition for allowance.

Respectfully Submitted,



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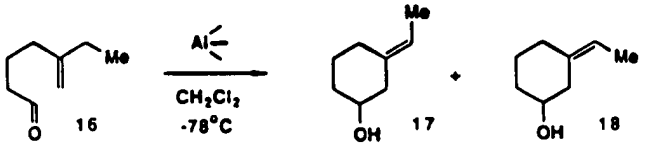
J. Am. Chem. Soc. 1990, 112, 9012-9013

EXHIBIT A

trans selectivity is best accounted for by the transition state **10** with both R and the carbonyl group equatorial rather than the alternative **9**.

Another interesting feature of MABR in the intramolecular ene reactions is the remote stereochemical control observed in the transformation of substrate **16** to *E*-olefinic alcohol **17** exclusively.[2]



Me_2AlCl : 63% (88 : 12)
MABR : 70% (100 : 0)

Supplementary Material Available: Experimental details of the Lewis acid preparation, ene reactions with MABR, and preparation of compounds **11** and **13** (2 pages). Ordering information is given on any current masthead page.

(5) The structure of **13** was confirmed by conversion to the known *trans*-decalin-1,3-diol (Grutzmacher, H.-F.; Tolkien, G. *Tetrahedron* **1977**, *33*, 221).

Probing Conformational Changes in Proteins by Mass Spectrometry

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Mass spectrometry has found wide application for the elucidation of the primary structures of proteins.[1] However, with the exception of topographical studies of membrane-bound proteins,[2] mass spectrometry has not previously been utilized to obtain information concerning the three-dimensional conformation of proteins. In the present communication, we describe the first use of mass spectrometry for probing conformational changes in proteins in a manner analogous to that employed in techniques like optical rotary dispersion, circular dichroism, and spectrophotometry.[3,4]

The new technique for probing the protein conformational changes makes use of electrospray ionization, which is a gentle method of ionization that produces intact multiply charged gas-phase ions from protein molecules in solution.[5,6] The multiply charged ions observed in the positive ion spectra are produced primarily as a result of proton attachment to available basic sites in the protein molecule. The availability of ionizable basic sites is determined by the conformation of the protein under the conditions of study, which include pH, temperature, and the presence of denaturing agents. In general, a protein in a tightly folded conformation will have fewer basic sites available for protonation compared to the same protein in an unfolded conformation. If the charge states of the gas-phase ions observed in the electrospray

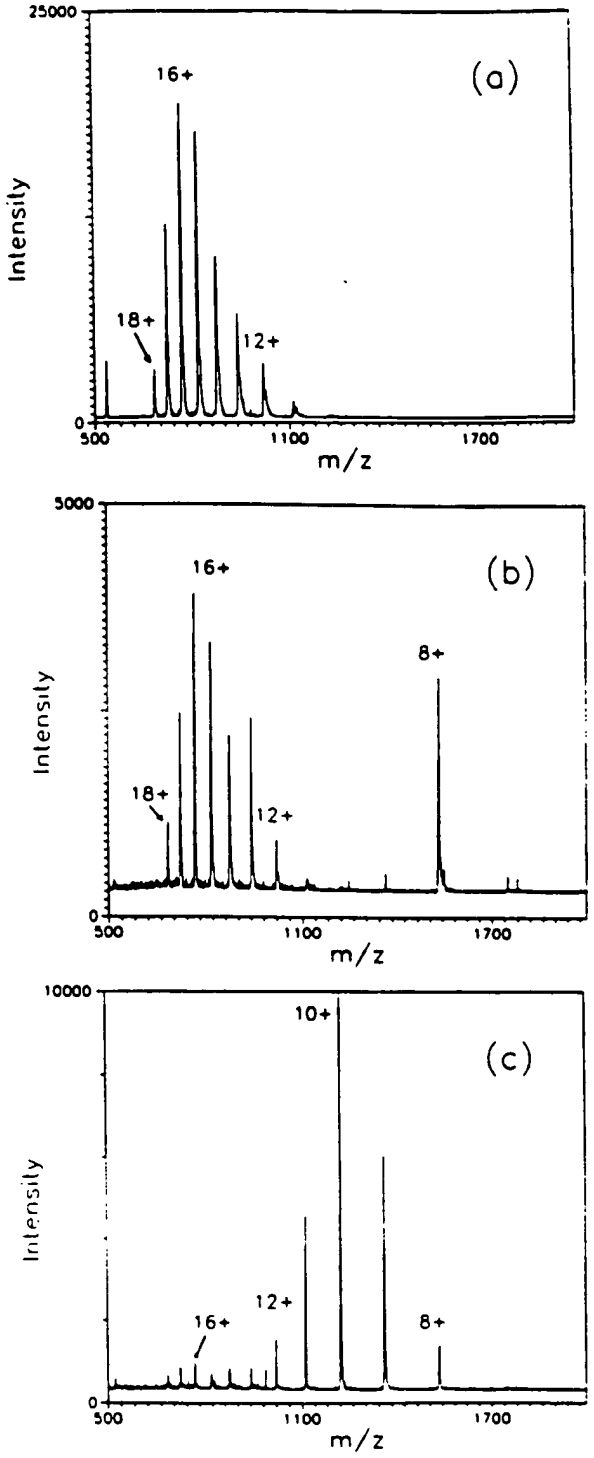

Figure 1. Electrospray ionization mass spectra of bovine cytochrome *c* obtained with different acetic acid concentrations in aqueous protein solutions. Protein concentration is 1×10^{-5} M: (a) 4% acetic acid, pH = 2.6, (b) 0.2% acetic acid, pH = 3.0, and (c) no acid, pH = 5.2. The labels on the peaks, *n*+, indicate the number of protons, *n*, attached to the protein molecule.

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EXIBIT A

mass spectrum reflect the charge states of the protein in solution, then the spectrum will yield information regarding the conformational state of the protein.

The mass spectra of bovine cytochrome *c*, shown in Figure 1, were obtained[7] by electrospraying aqueous solutions over a range of pH values (2.6–5.2) where the cytochromes *c* are known to undergo conformational changes.[9-15] The mass spectrum obtained from a solution at pH 2.6 (Figure 1a) exhibits eight peaks, each one corresponding to a different protonation state of cytochrome *c*. These protonation states range from 11+ to 18+ with 16+ being the most intense. Mass spectra of this type, exhibiting ions with a wide distribution of charge states and a single maximum, are typical of the electrospray ionization mass spectra of proteins that have been reported.[5,6,8] Surprisingly, as the pH of the sprayed solution was increased to 3.0, a second maximum with a protonation state of 8+ appears in the mass spectrum (Figure 1b). The width of this second distribution is narrow and is largely composed of the 8+ ion peak. Upon further increase of the pH to 5.2, the intensity of the distribution centered around 16+ decreases substantially and a third distribution centering around 10+ (with protonation states ranging from 8+ to 12+) is observed to dominate the spectrum (Figure 1c). Determination of molecular mass from the observed mass-to-charge ratios confirmed that all the peaks designated as 8+ to 18+ arise from intact bovine cytochrome *c*. We interpret the dramatic changes observed in the cytochrome *c* mass spectra (Figure 1) to result from differences in the conformational states of the protein in solution.

At low pH, the protein unfolds (state A) so that it can accept a large number of protons (Figure 1a). As the pH is raised, some of the cytochrome *c* molecules fold into a relatively tight conformation (state B) that can accept far fewer protons and produces a second distribution centered at the 8+ charge state (Figure 1b). The simultaneous observation of two discrete distributions of ions with no ions having intermediate charge states provides evidence for a highly cooperative transition between the two conformations. The tight conformation, B, can readily be converted into the highly charged unfolded state, A, by the addition of a denaturing agent such as methanol. Upon a further increase in the pH, virtually all the protein molecules are converted into a second folded conformation (state C) that can accept a larger number of protons than B but a smaller number than A.

The acid unfolding of cytochrome *c* has been extensively studied by various other techniques including acid–base titrations,[9] optical rotation,[10] spectrophotometry,[9,11-15] circular dichroism,[15] fluorescence,[15] NMR,[16] temperature jump,[12,17] and viscometry.[14,15] The existence of at least three conformational states in acidic conditions has been reported.[9,11,12,15,17] The results of the present investigation also indicate the presence of three distinct conformational states of cytochrome *c* for electrosprayed solutions in the pH range 2.6–5.2. In the electrospray ionization process, small highly charged droplets are initially formed that rapidly evaporate before gas-phase ions are finally produced. Because the evolution of the effective pH of the rapidly evaporating charged droplets is not known, a direct correlation between the presently observed and previously reported conformational states cannot be made.

The technique has also been applied to the investigation of conformational changes in horse cytochrome *c*, bovine ubiquitin, and yeast ubiquitin induced by changes in pH and by addition of organic solvents.[18] Dramatic changes in the charge distributions were observed in each case that could be correlated with changes in protein conformation. It is noteworthy that the charge distributions of proteins containing disulfide bonds have also been observed to be increased by reduction of the disulfide bonds.[19]

Our findings demonstrate the viability of a new physical method for probing conformational changes in proteins. In addition, these studies provide the basis for a better understanding of the roles of solvent composition and protein conformation in the degree to which proteins are ionized in the electrospray process.

Acknowledgment. This work was supported in part by Grants RR00862 and RR07065, Division of Research Resources, NIH, and GM38274, National Institute of General Medical Sciences, NIH. Insightful discussions with Dr. Ronald Beavis are gratefully acknowledged.

(7) The aqueous protein solutions (without the addition of any buffers) were electrosprayed at room temperature through a 150-μm-i.d. stainless steel syringe needle, whose tip was etched to provide a sharp conical shape. The electrospray ionization mass spectrometer used in the present investigations has been described earlier.[8] All the experiments were performed under identical conditions, except for the amounts of acetic acid added to the spray solutions and the flow rates, which ranged between 0.15 μL/min at pH = 2.6 and 1.0 μL/min at pH = 5.2.

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Surreptitious Involvement of a Metallacycle Substituent in Metal-Mediated Alkyne Cleavage Chemistry

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Received August 7, 1990

Potential applications of metallacycles in organic synthesis[1] and, more recently, in the area of electronic materials[2] have stimulated extensive research into the properties and reactivity of this compound class. A virtually untapped source of metallacycle reactivity lies in the chemistry of the ring substituents, particularly the α-substituents due to their close proximity to adjacent coordination sites.[3,4] Herein we describe the iridiacycle-mediated alkyne cleavage reaction represented by eq 1. Although such a transformation is rare,[5] and remarkable in its own right, labeling studies have revealed a novel mechanism that includes surreptitious involvement of an α-metallacycle substituent.

$$M + H_2O + HC{\equiv}CR' \rightarrow MCO + H_3CR' \qquad (1)$$

When a wet chloroform-d_1 solution of Ir(CR=CR-CR=CR)$(PPh_3)_2(NCCH_3)_2^+BF_4^-$ (**1**)[6] (R = CO_2CH_3, 9.5 mM) and methyl propiolate (95 mM) is maintained at 23 °C for

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EXHIBIT B

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 790–793, February 1993
Chemistry

Coexisting stable conformations of gaseous protein ions

(electrospray ionization/Fourier-transform mass spectrometry/hydrogen–deuterium exchange/protein conformation)

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Contributed by Fred W. McLafferty, October 19, 1992

ABSTRACT **For further insight into the role of solvent in protein conformer stabilization, the structural and dynamic properties of protein ions *in vacuo* have been probed by hydrogen–deuterium exchange in a Fourier-transform mass spectrometer. Multiply charged ions generated by electrospray ionization of five proteins show exchange reactions with ^2H_2O at 10^{-7} torr (1 torr = 133.3 Pa) exhibiting pseudo-first-order kinetics. Gas-phase compactness of the S–S cross-linked RNase A relative to denatured S-derivatized RNase A is indicated by exchange of 35 and 135 hydrogen atoms, respectively. For pure cytochrome *c* ions, the existence of at least three distinct gaseous conformers is indicated by the substantially different values—52, 113, and 74—of reactive H atoms; the observation of these same values for ions of a number—2, 7, and 5, respectively—of different charge states indicates conformational insensitivity to coulombic forces. For each of these conformers, the compactness *in vacuo* indicated by these values corresponds directly to that of a known conformer structure in the solution from which the conformer ions are produced by electrospray. S-derivatized RNase A ions also exist as at least two gaseous conformers exchanging 50–140 H atoms. Gaseous conformer ions are isomerically stable for hours; removal of solvent greatly increases conformational rigidity. More specific ion–molecule reactions could provide further details of conformer structures.**

The relationship between the dynamic structure of proteins in solution and their biological activity has been of long-standing research interest. Protein folding is probably the least well understood step in the sequence of transformations relating genetic information with its expression by protein function (1). Dramatic new ionization methods for mass spectrometry (MS) have made possible the formation of protein ions in the gas phase to measure molecular weight and primary sequence information (2–4), even on fmol samples (5, 6). Recent studies indicate that protein conformations in solution can affect the resulting charge distribution of the gaseous multiply charged ions formed by electrospray ionization (ESI) (7–9) and that even noncovalent complexes can survive ESI to form gaseous multiply charged ions (10–15).

Critical information concerning solvent effects on the conformation and dynamic properties of proteins has come from NMR (16) and from isotope-exchange experiments with ^2H_2O (17), including those before and during ESI/MS (18, 19). With an activation energy of 17–20 kcal/mol (1 cal = 4.184 J) (17), the H/^{2}H exchange rate depends on the pH (17), electrostatic effects (20), proximity of the solvent-accessible surface (21), and conformational flexibility with hydrogen bond cleavage and formation during local unfolding and folding (22). Studies of gaseous proteins should help delineate the role of solvent in stabilizing protein conformations, but such previous studies have been mainly theoretical (23) because of the lack of experimental approaches. We report here that conformations of gaseous multiply charged protein ions can be characterized by their H/^{2}H exchange kinetics, providing definitive evidence of distinct stable conformations that can coexist in the gas phase.

MATERIALS AND METHODS

Protein solutions were electrosprayed and the resulting ions were transferred and accumulated for 5 s (24) in the analyzer cell of a Fourier-transform ion-cyclotron-resonance mass spectrometer with a 6.2-T magnet. Designated preliminary experiments were run on a similar instrument with a 2.8-T magnet as described (5, 6). The ions were allowed to react for different time periods (separate runs) with ^2H_2O before excitation and detection.

Purified RNase A was a gift from H. A. Scheraga (Cornell University). RNase A S-alkylated with 4-vinylpyridine (VP-RNase) was obtained from RNase A by reduction with dithiothreitol in 4 M guanidinium isothiocyanate (0.1 M Tris·HCl, pH 8); thiol blocking by alkylation with 4-vinylpyridine; followed by quenching, dialysis, and lyophilization. Other protein samples were obtained from Sigma.

RESULTS AND DISCUSSION

Gaseous multiply charged ions were formed from equine cytochrome *c* (12.3 kDa) by ESI (2, 4–6). These were assumed to be pure (e.g., no noncovalently bound water molecules), as they exhibit the expected isotopic peaks (Fig. 1 *Inset*) and molecular mass values (5, 6). These ions were allowed to react with ^2H_2O (10^{-7} torr; 1 torr = 133.3 Pa) for increasing time periods (Fig. 1), with the increasing mass reflecting the number of ^{2}H atoms exchanged for H atoms versus time. A plot of these values (Fig. 2*A*) is consistent (exponential regression coefficients, ≈0.995) with pseudo-first-order exchange kinetics for each charge state (Fig. 2*B*). The left intercept also indicates the maximum number of hydrogens exchangeable by this process. Data observable at >1000 s and at higher pressure indicate that other H/^{2}H exchange processes must be slower by at least a factor of 20. The number of exchangeable hydrogens is nearly independent of charge state, although higher values have been achieved with other electrospray conditions (other than capillary temperature and voltage offset). However, electrospraying cytochrome *c* from $CH_3O^2H/^2H_3O^+$ solution indicates exchanges of 187 hydrogens (subtracting the $^2H^+$ species added in ionization); in this molecule, there are 198 hydrogens bound to heteroatoms, the type of hydrogen expected to undergo H/^{2}H exchange (17).

Surprisingly, the spectra of exchanged charge states 12+–14+ show (Fig. 1) peak splitting, indicating that two different

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Abbreviations: ESI, electrospray ionization; VP-RNase, S-alkylated RNase A; PA, proton affinity.

*To whom reprint requests should be addressed.

Submitted, Nucl. Instrum. and Meth. in Phys. Res. B (St. Malo) Best Regards! Carl T. Reimann

EXHIBIT C

Impacts of Polyatomic Ions on Surfaces: Conformation and Degree of Fragmentation of Molecular Ions Determined by Lateral Dimensions of Impact Features

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Massive, multiply-charged molecular ions produced by an electrospray ionization source are accelerated and directed onto flat target surfaces. Scanning force microscope (SFM) techniques are employed to examine the giant defects that are produced by *individual* incident ions. These defects appear as oblong hillocks, the widths of which indicate a typical distance over which the deposited energy density reaches a high enough level to permanently disrupt the surface structure. When the lengths of these hillocks exceed the widths, then the lengths yield qualitative information about the conformation of the incident molecular ions. Conversely, observation of low, small circular hillocks constitutes evidence for extensive fragmentation. Here, it is demonstrated in a simple visual fashion that electrosprayed poly(ethylene glycol) is easily fragmented by collisions with residual gas, and that electrosprayed *native* albumin molecules are partly denatured in the gas phase. Electrosprayed *denatured* albumin molecules appear in an extended, narrow configuration much longer than typical dimensions of the native molecule. Coulomb forces no doubt play a role in determining the structure of the gas-phase ions. These experimental results provide the first *direct* view of the conformation of electrosprayed molecules.

*Corresponding author.

EXHIBIT C

Abstract "Description '94" Conf. on Large Organic Ions Sun River Resort Oregon 3/27-31/94 EXHIBIT D

Monday, March 28, 199[redacted] 17:30 - 18:00

Collisional Excitation of Surfaces by Incident Energetic Polyatomic Ions: Recent Experimental Results and Theoretical Highlights

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The interaction between an incident energetic cluster ion and a surface can give rise to a numbe of unique phenomena due to the high density of deposited energy and the occurrence c cooperative collision sequences as the cluster impacts the surface. The physics of cluster-surfac impacts is only poorly understood at present; however, research on this topic has been stimulate by a number of practical issues. For example, cluster impacts have been demonstrated to provid a unique means of preparing[1] and modifying[2] surfaces. Also, in biological mass spectrometr massive biomolecular ions are detected by processes occurring when the analyte impacts detector surface. Two practical questions arise: How do detectors of biomolecular ions function Can these detectors be improved?[3,4,5] A further stimulus for mass spectrometrists has been th recent demonstration that cluster impact constitutes an exciting new means of desorbing large thermally labile molecules from surfaces as ions suitable for mass spectrometric analysis.[6]

In this contribution, a brief summary is given of results obtained using the Uppsala multiply charged macromolecular ion accelerator (MUMMA). In this project, surfaces are bombarded wit various protein ions accelerated to speeds around 50 km sec^{-1}. Resulting surface defects in the forr of craters and hillocks have been imaged with scanning probe microscopy techniques, giving clue about the conformation (shape) of electrosprayed protein ions. Secondary electron emission (SEE upon cluster impact has been studied, and trends observed in the data provide information abou various aspects of the cluster-surface interaction. SEE also provides useful information about th mass of the analyte. Finally, a preliminary experiment on ionic desorption induced by inciden cluster ions has been performed. The yield of secondary ions is sublinear in impact kinetic energ and seems to depend on molecular conformation. The MUMMA results obtained so far are briefl compared with results from several parallel experiments carried out by other research groups.

At present, our understanding of energetic cluster-surface interactions is strongly guided b theoretical efforts. In particular, increasingly sophisticated molecular dynamics simulation provide a wealth of clues on the slowing-down of impacting clusters and on transient high energy density conditions existing during and after impact. In this contribution, a few theoretica highlights are given, where appropriate. Considering the existing experimental and theoretica state-of-the-art, a number of open questions remain which form the basis for a new generation o experiments.

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A QUADRUPOLE/TOF MS/MS EXPERIMENTS TO STUDY EFFECTS OF IMPACT OF MACROMOLECULAR IONS ON SURFACES

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A "multiply-charged macromolecular accelerator" (MUMMA) is being constructed at Uppsala University for studies of macromolecular ion impacts on surfaces [1,2]. An electrospray ion source and a quadrupole mass spectrometer (MS1) are employed to generate and select positive ions from peptides with mass M from 0.5 to 66.3 kDa and charge Z up to 60, in the M/Z range below 2000. The system is modified by addition of ion optics for transport of the generated ions towards an external target held at a potential U_a of up to - 20 kV. The U_a used in this study was -10 kV, corresponding to primary ion energies Z·10 keV(up to 600 keV for albumin projectiles). A straight time-of-flight (TOF) spectrometer (MS2) has been used for mass analysis of the ejected negative secondary ions as a result of a single macromolecular ion impact on different organic and inorganic targets (Fig.1). The initial experiments, reported here, were performed at a typical pressure in the experimental chamber of $5 \cdot 10^{-7}$ Torr maintained by an oil diffusion pump (an UHV-style experimental chamber is currently under construction).

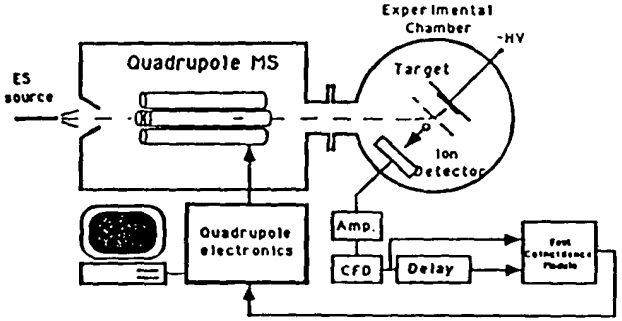

Fig.1 A quadrupole/TOF MS/MS experimental set-up.

Two modes of MS/MS data collection have been developed. Initially MS1 was tuned to a particular M/Z and complete TOF spectra were collected by MS2 (Fig. 2). The negative ion TOF spectra have been obtained using as a start signal either the ejected secondary electrons or H^- ions. Although different target materials have been tested (valine, CsI, bare stainless steel, etc.), 95 to 98 % of the detected secondary ions were not target-specific. Spectra contained mainly peaks of low mass hydrocarbon ions which might have originated from the contamination layer on the target and/or from the projectile. That mode of operation has been employed for estimates of the secondary ion multiplicities (i.e. number of registered secondary ions with different masses per impact event) as a function of different projectile parameters [3]. Our results demonstrate that the average multiplicity of secondary ions ejected in macromolecular impact events scales sublinearly with the projectile velocity [3].

In the second mode fast coincidence electronics is employed to record yields of secondary ions within a predetermined M/Z window (i.e. MS2 collecting ions within a specified arrival time in the TOF spectrum) while the quadrupole (MS1) is scanned through M/Z 500 to 2000, thus resulting in a spectrum of the projectile ions in different charge states (Fig. 3). The secondary electrons emitted during the impact can be magnetically deflected and thus the mass spectrum will contain only secondary ions and/or

fragments from the primary ion projectile. We note the applicability of this approach for resolving the individual components of an electrosprayed mixture (of e.g. peptides), provided that component-specific fragments are detected in the secondary ion TOF spectrum. By choosing a time window for that particular ion the projectile ion spectrum for that component will be accordingly enhanced. So far we have not been able to demonstrate that approach with this particular set-up since we cannot pinpoint a fragment, specific for the primary ion, most probably due to contaminations on the target.

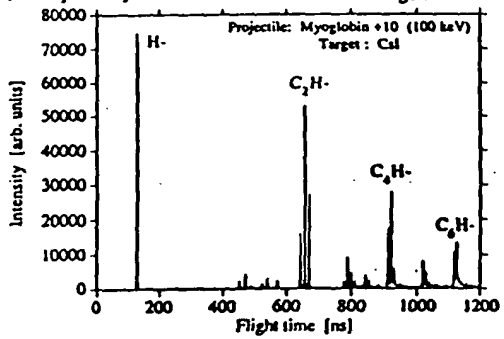

Fig.2 TOF secondary ion spectrum: 100 keV myoglobin ions as projectiles.

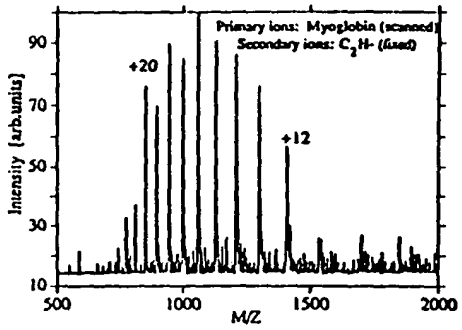

Fig.3 MS/MS mass spectrum of myoglobin, secondary ions: C_2H^-.

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EXHIBIT F

Cluster-Impact Fusion

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(Received 9 June 1989)

Deuteron-deuteron fusion, detected via the 3-MeV protons produced, is shown to occur when singly charged clusters of 25 to 1300 D_2O molecules, accelerated to 200 to 325 keV, impinge on TiD targets. The energy and cluster-size dependence of the fusion rate are discussed. The fusion events are shown to originate from the cluster-ion impacts rather than from D^+ or D_2O^+ ions in the beam. The observed rates may be correlated with the compressions and high energy densities created in collision spikes by cluster-ion impacts.

PACS numbers: 79.20.Rf, 25.45.−z, 47.40.Nm, 52.50.Lp

The impact of accelerated cluster ions on solid surfaces has been shown to produce very high transient particle and energy densities.[1] The experiments reported in this Letter show that singly charged, heavy-water cluster ions containing between 25 and 1300 D_2O molecules and accelerated to energies up to 325 keV can, on impact with titanium deuteride targets, generate thermonuclear D-D fusion reactions.

The apparatus used for the experiments is shown schematically in Fig. 1. The ion source and the techniques for mass analysis and for acceleration of water cluster ions have previously been described in detail.[2] Water molecule ions in helium carrier gas were generated in a corona discharge. The weakly ionized gas mixture was expanded through a nozzle and skimmer into a high-vacuum system. As discussed previously,[2,3] a combination of mass analysis and careful determination of secondary-electron yields produced by impact of cluster ions on a target suffices to determine the m/e ratios of the ions and to count the number and kind of atoms in the cluster.

The cluster ions prepared by these techniques were subjected to quadrupole mass analysis[2] and electrostatic focusing before introduction into a Cockroft-Walton accelerator approximately 1 m long. After acceleration, ions passed through an aperture into a vacuum chamber containing a TiD target of about 1-cm^2 surface area at −45° to the beam axis and approximately 1.5 cm from a 300-mm^2 Ortec "ruggedized" silicon solid-state detector. The active surface of the detector was covered with a 50-$\mu g/cm^2$ layer of aluminum.

The cluster beam current was monitored by measurement of secondary electrons ejected from the TiD target (i.e., measurement of apparent positive-ion current to the target). Cluster-ion intensity measurements are subject to error when a small fraction of the ions make grazing collisions with the edges of the column exit aperture. Such collisions produce low-mass fragments [e.g., 300-eV deuterons from 300-keV $(D_2O)_{100}$ clusters] that can eject secondary electrons from the target but do not have enough energy to produce D-D reactions. Careful attention to optimization of secondary-electron distributions is required to ensure proper beam focusing, particularly when the beam energy is varied. Relative values of pri-

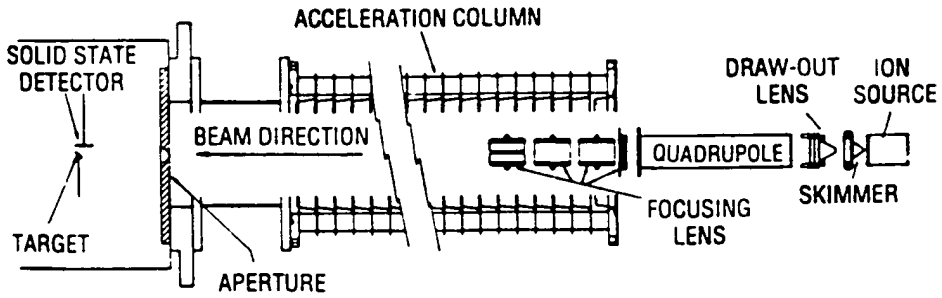

FIG. 1. Schematic diagram of the apparatus. A mixture of D_2O and He gas is ionized in a corona discharge in the ion source and expanded through a supersonic nozzle into a differentially pumped region between nozzle and skimmer. Cluster ions pass through the skimmer, are extracted with 10–50 eV by the draw-out lens, and mass analyzed in the quadrupole mass spectrometer using a low-frequency (−292 kHz) power supply. The mass-analyzed ions are focused and then accelerated in a Cockroft-Walton column. Ions traverse an −1-cm aperture and impact on a TiD target attached to a Keithly picoammeter. A silicon solid-state detector −1 cm from the target is used to measure protons, tritons, and x rays produced by cluster impact on the TiD target.

EXHIBIT G

STUDIES OF A HIGH INTENSITY ELECTROSPRAY ION SOURCE CONNECTED TO A HIGH MASS POST ACCELERATION ION DETECTOR

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Electrospray ionization (ESI) has been shown to generate multi-charged ions which can be identified by determination of mass to charge ratios.[1] Mass or charge determination in electrospray mass spectra requires sufficient resolution to separate ions carrying different charges. With sufficient resolution of very high molecular weight ions, the identification of masses of unknown samples may be difficult or impossible. If ions are subjected to post-acceleration followed by determination of secondary electron pulse spectra,[2] ion masses and charges can be established. Secondary electron pulse spectra are obtained by accelerating electrons generated from a detector dynode and determining the energy deposited by the electron pulse into a solid state detector. Electron pulses measure the electronic excitation deposited in the detector dynode. This excitation is related to the sum of the atomic masses in a molecular ion and its velocity.[3] Ion velocities can be calculated directly from ion mass to charge ratios and ion kinetic energies. Thus secondary electron yields can provide the information necessary for computation of ion masses.

The application of this technique of mass determination to ESI spectra of polyethylene glycols[4] provides evidence of decomposition for polymers with

molecular weights greater than 20,000 amu. The conclusion that PEG polymers with mass 5,000,000 amu can be generated with 4000 charges must be seriously questioned. The possibility that decomposition occurs in the acceleration column after mass analysis was tested by using energy selector plates to filter lower energy ions before impact on the detector dynode. The results of this test indicated decomposition prior to mass analysis.

Secondary electron spectra can reveal the presence of ions with radically different mass or charge in spectra which show unresolved ions with similar or identical mass to charge ratios. The use of secondary electron pulse spectra for mass determination of multi-charged ions will be demonstrated in studies of the monomer and dimer of apoferritin (molecular weights of 480,000 and 960,000 and with m/e values of approximately 13,500 and 19,000 respectively). Multi-charged high molecular weight solvent clusters containing methanol and water were also detected and the charge determined to be 21 on a mass of 294,000 amu. The larger linear PEG polymers were the only cases in a variety of studies where evidence was found for ion decomposition.

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EXHIBIT H

Comparisons of Low Versus High Energy Collisional Activation for the Sequence Analysis of Large Peptides

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Tandem mass spectrometry (MS/MS) offers several advantages over traditional Edman degradation in the sequential analysis of proteins, including the abilities to sequence peptides present in mixtures, identify modified amino acids, sequence peptides with blocked N-termini, and significantly reduce sample consumption in favorable cases. A current shortcoming of MS/MS sequencing of singly charged ions is limited mass range of peptides (<3.5 kDa) that are amenable to this approach. A possible explanation for this upper mass limit is the inverse relationship between the size of the precursor ion and the amount of energy deposited during the collisional activation (CA) process. Consequently, only a few singly charged peptide ions over 3.0 kDa have been sequenced by MS/MS. The recent invention of electrospray ionization (ESI) has greatly increased the mass range of biopolymers that can be measured by mass spectrometers. These multiply charged ions appear to possess higher internal energies due to intramolecular coulombic repulsions than the singly charged ions. Because of their higher internal energies, multiply charged ions of large proteins, up to 66 kDa, have been fragmented by CA.

In this report, we discuss our observations in the sequential analysis of the $(M+5H)^{5+}$ ion of pancreatic polypeptide bovine (PPB) using a Sciex (Ontario, Canada) API III quadrupole mass spectrometer and a JEOL (Tokyo, Japan) HX/HX 110A tandem four-sector mass spectrometer equipped with an array detector. The MS/MS spectrum (Fig. 1) of the $(M+5H)^{5+}$ ion of PPB was obtained on the Sciex instrument. In this experiment a peptide solution of 50 pmol/μL was infused into the MS at a flow rate of 5 μL/min. The ion spray needle was held at 5 kV and the orifice voltage was 70 V. This spectrum gives only partial sequence information, presumably due to the low laboratory collision energy. On the other hand, high energy CA of $(M+5H)^{5+}$ ion of PPB allowed the complete sequence confirmation of PPB (Fig. 2) to be determined by the four-sector instrument. This spectrum was obtained by infusing 50 pmol/μL at a flow rate of 1 μL/min into an Analytica Branford ESI source. The same quantity of peptide was used on both the quadrupole and sector instruments.

Acknowledgment

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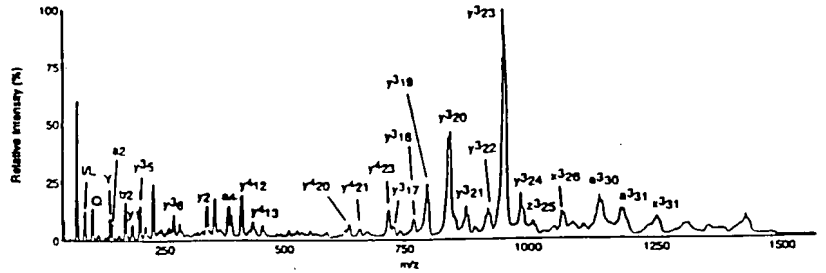

Fig 1. Low energy MS/MS spectrum of $(M+5H)^{5+}$ ion of pancreatic polypeptide, bovine FW 4225.8 (A-P-L-E-P-EY-P-G-D-D-A-T-P-E-Q-M-A-Q-Y-A-A-E-L-R-R-Y-I-N-M-L-T-R-P-R-Y-NH_2)
Laboratory collision enery - 650 eV ;Collision gas - Xenon

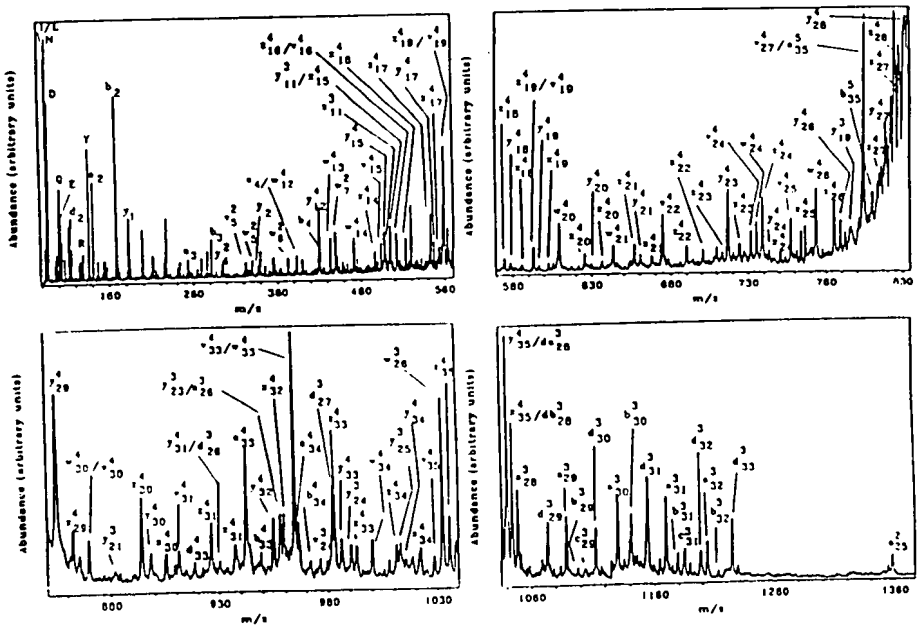

Fig 2. High energy MS/MS spectrum of $(M+5H)^{5+}$ ion of pancreatic polypeptide, bovine
Laboratory collision energy - 5000 eV ; Va-7kV and Vc-6kV ;Collision gas - Helium

EXHIBIT I

Determination/Verification of Nucleotide Sequences by ESI/FTMS

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The immensity of the Human Genome Project has generated an intense search for fast methods of nucleotide sequencing and sequence verification. Due to the fragile nature of the phosphodiester backbone and the accompanying difficulty in volatilizing nucleotides, mass spectrometry (MS) has not yet played a significant role in their sequencing. The recent introduction of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI) have made possible molecular weight determination of both oligonucleotides and proteins by MS. With ESI, McLuckey et al. have obtained full sequence for oligonucleotides as large as an 8-mer with MS^3 on their ion trap instrument, and provided a valuable understanding of the fragmentation mechanisms upon collisional activation.[1] Here, ESI coupled with Fourier transform mass spectrometry (FTMS) achieves sufficiently high mass accuracy (10 ppm routinely) and resolving power to provide base composition and sequence information for DNA with as many as 50 bases. The high mass accuracy obtained with FTMS dramatically decreases the number of possible base compositions for a given MW, and is especially useful for these PCR primer-sized oligos. As an example, for the 25-mer A_5T_{20} (7584.253 Da) there are 20 possible degeneracies in composition within ± 0.5 Da; this is reduced to 3 degeneracies when measured with 2 ppm precision, easily obtained with internal calibration. Nozzle-skimmer (NS) dissociation yields fragments ions that result in complete sequence determination for a 14-mer (Figure 1), complete sequence verification for a 25-mer,[2] and extensive sequence verification (19 bases) for a 50-mer DNA.

Without prior desalting, highly accurate MW data is not easily obtained for larger (>50 bases) nucleotides due to the high level of cation adduction. Stults and Marsters used EtOH precipitation to replace Na^+ with NH_4^+, and reduced the level of adduction such that an accurate molecular weight could be obtained for a 48-mer.[3] Reverse-phase HPLC desalting is even more effective at adduct removal, as demonstrated by spectra of a 76-mer $tRNA^{phe}$ (25 kDa, Figure 2) and a 100-mer DNA (31 kDa, Figure 3), in which the non-adducted molecular ion isotopic cluster is larger than any cation adducted cluster. The resulting improvement in signal for $tRNA^{phe}$ makes possible the detection of an expressed minor variant 14 Da lighter than the predominant sequence.

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2. Little, D. P.; Chorush, R. A.; Speir, J. P.; Senko, M. W.; Kelleher, N. L.; McLafferty. To appear in J. Am. Chem. Soc., June 15, 1994.

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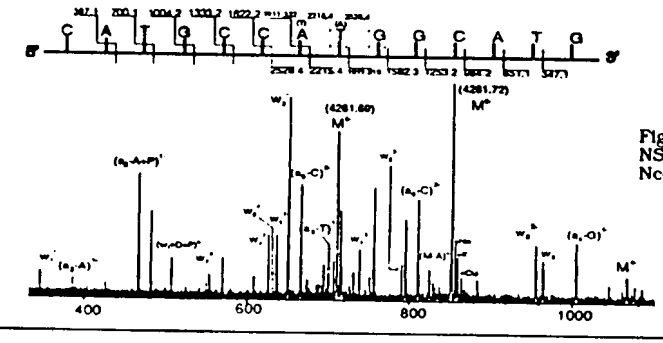

Figure 1. NS dissociation of Nco I (4261.75 Da).

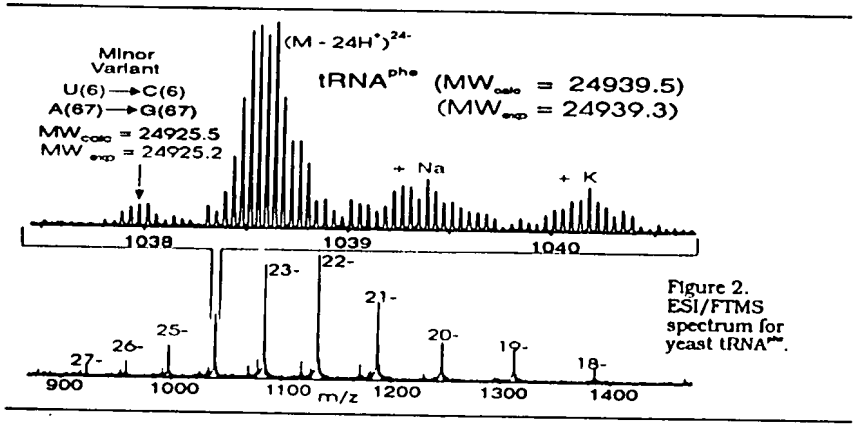

Figure 2. ESI/FTMS spectrum for yeast $tRNA^{phe}$.

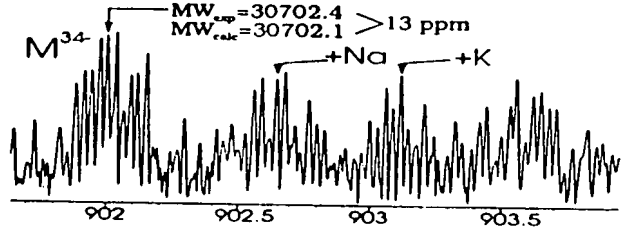

Figure 3. $A_{50}T_{50}C_{10}G_{10}$.

Collision Cross Sections for Protein Io

EXHIBIT J

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A method for the determination of cross sections for gas-phase protein ions, based on the energy loss of ions as they pass through a collision gas, is described. A simple model relates the energy loss to the number of collisions and hence the cross section. Results from a Monte Carlo model that support the validity of this approach are described. Experimental cross sections are reported for motilin, ubiquitin, cytochrome *c*, myoglobin, and bovine serum albumin. Cross sections range from approximately 800 Å^2 for motilin to approximately 14,000 Å^2 for bovine serum albumin and generally increase with the number of charges on the ion. Cytochrome *c* ions from aqueous solution show somewhat smaller cross sections than ions formed from solutions of higher organic content, suggesting that the gas-phase ions may retain some memory of their solution conformation. *(J Am Soc Mass Spectrom 1993, 4, 616–623)*

The development of electrospray and related ionization techniques has allowed, for the first time, the formation of many gas-phase protein ions [1]. Physical properties of these ions, such as size or conformation, are largely unknown. Collision cross sections can give one measure of the ion size (which may be related to conformation), but little is known about collision cross sections for these ions. Smith and Barinaga [2] reported dissociation cross sections of approximately 1000 Å^2 (10^{-13} cm^2) for cytochrome *c* ions. A study of collision "focusing" in radiofrequency (RF) quadrupoles led to the speculation that collision cross sections must be approximately 1000 Å^2 or more for such ions [3].

This report describes a novel method for the determination of collision cross sections for gas-phase protein ions. The loss of axial energy of an ion as it passes through a collision cell, containing an inert gas, is measured. A simple model gives the average energy loss in a single collision so that from the energy loss, the total number of collisions and hence cross section can be calculated. The use of the energy loss of ions to determine physical properties of ions (or the target) is not new (see, e.g., Bohr [4]) but to our knowledge, this is the first application to gas-phase ions of biomolecules. It is shown that for the ions studied, cross sections are approximately 10^3–10^4 Å^2 and that the method may find use for studies of conformations of these ions. Described here is the experimental procedure, a simple model for the energy loss process, a Monte Carlo simulation of ion energy distributions, and collision cross sections for ions formed from motilin, ubiquitin, cytochrome *c*, myoglobin, and bovine serum albumin.

Experimental

All experiments were performed on a PE-Sciex API III triple quadrupole mass spectrometry system, shown schematically (with notation) in Figure 1. Ions formed by pneumatically assisted electrospray (ion spray) enter the vacuum chamber through a small orifice and pass through an RF quadrupole (Q0) to the first analyzing quadrupole (Q1), also operated in RF-only mode for this report. The potentials applied to the system are shown in Table 1. Ions enter the first RF quadrupole (Q0) at the potential of the orifice but have a sufficient number of collisions with the gas expanding from the orifice that their energies are moderated to a few volts or less in Q0. Therefore at Q2, the ions appear to be formed at a potential close to the Q0 rod offset. Because the potential difference between the Q0 and the collision cell (Q2) rod offset voltages was 10 V, ions entered the collision cell with an energy of approximately 10*i* eV, where *i* is the number of charges on the ion (center-of-mass energies were typically 0.1–0.8 eV). With no collision gas added, stopping potentials were 10 ± 0.5 V, in accord with this interpretation. Under these conditions, no collision-induced dissociation was seen. Quadrupole Q3 was operated in mass-resolving mode. Energy distributions of ions leaving Q2 were determined approximately from stopping curves obtained by increasing the Q3 rod offset voltage in steps of 1.0 V until the ion signal was attenuated by approximately three orders of magnitude. Ion energy spreads (at 10%) were generally approximately 1 eV; exceptions were some ions produced from cytochrome *c* (see

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EXHIBIT K

Surface-Induced Dissociation of Multiply Protonated Peptides

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We report here surface-induced dissociation spectra of three multiply charged peptides: doubly protonated angiotensin I, doubly protonated renin substrate, and triply protonated melittin. For comparison, the collision-activated dissociation spectra of renin substrate and melittin are also presented. The spectra show that surface-induced dissociation provides structural information on multiply charged peptides at the picomole per microliter sample concentrations compatible with electrospray ionization. For multiply protonated angiotensin I, renin substrate, and melittin, surface collisions (100–165 eV) favor a limited number of fragmentation pathways, which are the same as those favored in collision-activated dissociation experiments. *(J Am Soc Mass Spectrom 1992, 3, 859–862)*

In the case of singly protonated peptides produced by liquid secondary ion mass spectrometry (LSIMS), gas-phase collisional excitation of large peptides (< 3000 Da) produces sufficient fragmentation for structural analysis [1]. Extension to larger peptides is limited by losses in desorption and ionization efficiency and by the partitioning of a limited amount of internal energy to a large number of vibrational modes. Electrospray ionization (ESI) has gained considerable attention because it is an efficient means of generating multiply charged ions from large biomolecules, including proteins [2–6]. Although the fragmentation mechanisms for formation of multiply charged peptides have not been fully elucidated, low-energy gas-phase collisional activation of mass-selected multiply protonated peptides has been shown to provide structural information [3]. Collision-activated dissociation (CAD) of peptides between the ESI skimmer cone and capillary can provide an additional method for obtaining sequence information [7, 8]. Surface-induced dissociation (SID) is an alternative means of dissociating ions; several investigators have reported SID spectra of singly charged peptides produced by LSIMS [9–13]. We report here SID spectra of multiply charged peptides and compare the spectra with those obtained by CAD.

Experimental

The instruments used in this investigation were a simple, inexpensive dual quadrupole mass spectrometer specifically designed for ion/surface studies [14] and a triple quadrupole mass spectrometer (Finnigan TSQ70, San Jose, CA). Experimental details for the triple quadrupole mass spectrometer have been reported previously [15]. The SID instrument consisted of two Extrel (Pittsburgh, PA) quadrupoles (m/z range 0–4000 Da) arranged at 90°, with a surface positioned to intersect the ion optical path of each quadrupole. The angle of the incident beam was 50° with respect to the surface normal. The surface used in this investigation was stainless steel, although alternative surfaces are being investigated [16]. Data were acquired and processed with a Teknivent/Vector Two data system (Maryland Heights, MO).

Electrospray ionization on the SID instrument was accomplished by using a modified version of the recently published electrospray designs of Chowdhury et al. [17] and Papac et al. [18]. The samples were dissolved in a 1:1 methanol:1% acetic acid solution at final concentrations of 10–30 pmol/μL. Samples were sprayed with a syringe pump through a syringe needle (4–5 kV) toward a metal capillary (170–200 V) at a rate of 2μL/min. A heater wire in fiberglass sleeving was wrapped around the metal capillary to thermally desolvate the ions. The multiply protonated peptides were mass selected by Q1 and allowed to collide with the surface at a selected laboratory collision energy. The product ions were analyzed by Q2. The laboratory collision energy is determined by (1) the potential difference between the skimmer cone and the surface and (2) the charge state of the ion. For simplicity, the potential difference between the skimmer cone and surface will be listed as ΔV; the kinetic energy of the collision is determined by multiplying ΔV by the charge state. Good quality SID spectra can be obtained

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